Exploring the Influence of Arbuscular Mycorrhizal Symbology on the Antioxidant Potential of Liverwort Asterella multiflora: A Comprehensive Study on Rhizoid and Thallus Anatomy

Mamta Verma¹, Shabnam¹, Sandeep Kotwal² and Anupam Kumar³*¹

¹Department of Botany, School of Bioengineering and Biosciences, Lovely Professional University, Phagwara-144402, Punjab, India; ²Department of Botany, Government Degree College Doda-182202 affiliated to University of Jammu, Jammu and Kashmir, India; ³Department of Biotechnology, School of Bioengineering and Biosciences, Lovely Professional University, Phagwara-144402, Punjab, India

E-mail/Orcid Id:

MV; vermamanta52@gmail.com, https://orcid.org/0009-0006-8251-6896; S; shabnam.28725@lpu.co.in, https://orcid.org/0000-0003-2949-3518; SK; s.kotwal@yahoo.com, https://orcid.org/0000-0002-7995-5437; AK; anupam.kumar167@gmail.com, https://orcid.org/0000-0002-6608-3860

Abstract: Arbuscular mycorrhizal (AM) symbiosis is a vital ecological interaction between plants and fungi that enhances nutrient uptake and plant resilience. While extensively studied in vascular plants, AM symbiosis in liverworts remains relatively unexplored. Six populations of Asterella multiflora were collected throughout the year to scan for arbuscular mycorrhizal colonization and enzymatic antioxidant activity. Percent mycorrhizal colonization was measured in smooth as well as tuberculated rhizoids. Anatomical detail of the thallus was also observed. Arbuscules were observed in the cells of the storage zone of the thallus. Enzymatic antioxidant activities, i.e., superoxide dismutase (SOD), guaiacol peroxidase (GPOX), catalase (CAT), ascorbate peroxidase activity (APOX) and glutathione reductase activity (GR) were calculated. Maximum enzyme activity was observed in ascorbate peroxidase, which calculated 1.92-2.09 UA/mg protein, while catalase showed minimum activity of 0.01 UA/mg protein. A positive correlation was observed between enzyme activities and percent mycorrhizal colonization. This study delves into AM symbiosis in liverworts, focusing on Asterella multiflora and investigates the impact of AM colonization on enzymatic antioxidant activities, i.e., superoxide dismutase (SOD), guaiacol peroxidase (GPOX), catalase (CAT) ascorbate peroxidase activity (APOX) and glutathione reductase activity (GR). Antioxidants play a crucial role in stress tolerance and plant health, making them central to understanding the implications of symbiotic relationships.

Keywords: Asterella multiflora, Antioxidants, Arbuscular Mycorrhiza, Liverworts

Introduction

Bryophytes comprise three major groups: hepatics (liverworts), hornworts and mosses. They are an extraordinary group among embryophytes and have high species diversity that is only next to angiosperms (Christenhusz and Byng, 2016). They are very small in size and lack true roots and a vascular system. In the life cycle of bryophytes, the gametophyte is dominant and free-living, while the sporophyte partially or wholly depends on the gametophyte for water and nutrition supply. The gametophytes of bryophytes show a thalloid organization or differentiate into root-like rhizoids, stem-like colloids and leaf-like phylloid structures. The bryophytes are very interesting miniature plants that lie between green algae and vascular plants and, thus, are considered to arise soon before the origin of land plants (Bowman et al., 2016). Bryophytes play a significant role in the ecosystem in many aspects, viz., water retention, ecological indicators, soil improvement and nutrient cycling (Rousk et al., 2017; Glime, 2020; Xiao and Bowker, 2020). They are the economically important amphibians of the plant kingdom. Furthermore, their simplicity and evolutionary significance make them

*Corresponding Author: anupam.kumar167@gmail.com
valuable model organisms for scientific research (Yadav et al., 2023).

During the evolution of land plants, liverworts are among the pioneers in adapting to desiccant and nutrient-poor habitats with elevated ultraviolet radiation. A great amount of ecological success has been achieved by bryophytes in diverse habitats, from aquatic to desert and from arctic to tropical. As the first group to exploit land plant habits, Bryophytes must have acquired many morpho-anatomical, physiological and reproductive modifications that confer selective adaptations under changing environmental conditions. One of these adaptations was the development of arbuscular mycorrhizal symbiosis with fungi before the evolution of true mycorrhizae (Redecker et al., 2000). The apparent lack of or at least poor development of roots in the earliest bryophytes, such as plants, in tandem with the scarcity of variable nutrients, especially phosphorous, in the rudimentary soils, necessitated the evolution of AM symbiosis (Cairney, 2000; Antoine et al., 2021; Veresoglou et al., 2022). Mycorrhizae represent a range of mutualistic associations between soil fungi and plant roots. Although bryophytes do not have true roots, they have rhizoids, many of which are associated with mycorrhizal fungi (Russell and Bulman, 2005; Duckett et al., 2006; Ligrone et al., 2007; Pressel et al., 2008; Verma and Langer, 2011; Liepinia, 2012). Rimington et al. (2018), revealed that Glomeromycota structures in liverworts encompass features of both Arum and Paris types. Furthermore, Kobae et al. (2019) isolated arbuscular mycorrhizal fungi from the young thallii of Marchantia paleacea, reinforcing the significance of these symbiotic relationships in liverwort ecology. Rimington et al. (2020) concluded that 78% of hornworts and lycophytes, and up to 100% of certain species, engage in fungal symbiosis, with members of Mucoromycotina and Glomeromycotina playing vital roles in these interactions. The reactive oxygen species, at higher concentrations, have the capability to produce oxidative stress, which may be responsible for many degenerative diseases, ageing, apoptosis and food rancidity (Pisoschi and Pop, 2015). Antioxidants neutralize the reactive oxygen species. The major sources of antioxidants are phytochemicals derived from plants. It has been observed that plant-derived natural antioxidants have a more progressive effect on the human body than synthetic antioxidants. In recent years, the importance of biologically active compounds from plants has gained significant interest due to their ability to combat diseases caused by oxidative stress (Dixit, 2021). Higher plants, particularly angiosperms, are commonly explored and used as antioxidant sources. However, there is much work going on in foreign countries in exploring bryophytes as antioxidant sources, but only reports in India (Singh et al., 2006; Krishnan and Murugan, 2013; Sharma et al., 2015), which have a strong defense mechanism to survive in highly diverse habitats and have a reservoir of many phytochemicals, are not well studied for antioxidant profiles.

The present study aims to delve into the intricate relationship between Asterella multiflora and arbuscular mycorrhizal fungi, specifically investigating this symbiosis’s influence on the liverwort’s antioxidant potential. Antioxidant defenses are crucial components of a plant's response to environmental stressors (Bhatta et al., 2023; Rami et al., 2023) and understanding how AM symbiosis may modulate these defenses in non-vascular plants adds a novel dimension to our comprehension of plant-fungal interactions.

Furthermore, our comprehensive examination will focus on the anatomical aspects of both the rhizoids and thallus of Asterella multiflora, shedding light on potential structural adaptations induced by AM symbiosis. By elucidating the intricate interplay between this liverwort species and arbuscular mycorrhizal fungi, we aim to contribute to the broader understanding of symbiotic associations in non-vascular plants and their implications for plant health and adaptation in various ecosystems.

This investigation expands our knowledge of liverwort biology and underscores the importance of considering diverse plant-fungal interactions in the context of plant antioxidant responses. The findings of this study may have implications for ecological restoration efforts and provide valuable insights into the co-evolutionary dynamics between liverworts and arbuscular mycorrhizal fungi.

Methods
Liverwort samples (six populations) growing along one-kilometre stretch were collected throughout the year from the artificial embankment near the Mahamaya temple in Jammu. One hundred and twenty thalli were observed during the year to conclude for Arbuscular mycorrhizal colonization. To characterize the species composition of the bryophyte community, a Nikon Eclipse 400 camera was used for imaging, and morphological identification followed the criteria established by Rawat et al. (2015).

Quantification of AM
For assessing arbuscular mycorrhizal fungi (AMF) colonization, sections of gametophytes from each
samples were taken, with 50 sections from senescent (older) and 50 sections from green (healthy) gametophytes. These samples were cleaned of soil particles and substrate debris by washing with tap water. The gametophyte sections were then preserved in 70% ethanol overnight and treated with a 1% KOH solution for 20 minutes at 80°C. Afterward, they were kept in 1% HCl for 10 minutes (50°C). The sections were stained in 0.05% trypan blue and kept for 20 minutes (60°C), following the method described by Gottet et al. in 2018. The stained sections were examined under a microscope to observe distinctive AMF structures. Additionally, fungal spores were isolated using wet sieving and decanting method, modified from a previously established procedure. These spores were used for Arbuscular Mycorrhizal Fungal morphological identification based on taxonomic features described in the works of Paczkowski in 2015 and Oehl et al. in 2015. The degree of AM colonization was determined by the method given by Nicolson (1967) as follows:

Percentage mycorrhizal colonization (PMC) = (No. of rhizoids colonized / Total no. of rhizoids scanned) x 100

The bryophyte samples were submitted to the Herbarium of Botany, University of Jammu.

Preparation of Plant Extract for determining antioxidant activities

In a prechilled pestle and mortar (in an ice box), 0.5 gm of liverwort tissue was homogenized in 3 ml of 0.1M potassium phosphate buffer. The mixture was then centrifuged at 13000 rpm for 20 minutes. Protein content and the activity of antioxidant enzymes were estimated using the supernatant. These biochemicals were estimated by the methods described by the following authors (Table 1):

Table 1. Methods used to determine enzymatic antioxidants

<table>
<thead>
<tr>
<th>Sl.</th>
<th>Biochemicals</th>
<th>Method used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Protein</td>
<td>Lowry method</td>
</tr>
<tr>
<td>2.</td>
<td>Catalase (CAT)</td>
<td>Aebi, 1984</td>
</tr>
<tr>
<td>3.</td>
<td>Guaiacol peroxidase (GPOX)</td>
<td>Putter, 1974</td>
</tr>
<tr>
<td>4.</td>
<td>Superoxide dismutase (SOD)</td>
<td>Kono, 1978</td>
</tr>
<tr>
<td>5.</td>
<td>Ascorbate peroxidase (APOX)</td>
<td>Nakano and Asada, 1981</td>
</tr>
<tr>
<td>6.</td>
<td>Glutathione Reductase (GR)</td>
<td>Nordhoff et al., 1993</td>
</tr>
</tbody>
</table>

Results and Discussion

Both tuberculated and smooth-walled rhizoids were colonized by fungi in all studied samples, but the frequency of mycorrhizal colonization was substantially higher in the smooth-walled type of rhizoids. Aseptate fungal hyphae were also. Typically, fungi grow by making parallel hyphal strands (Fig. 2b) that run across the rhizoids. Rhizoids typically contained Y- and H-connections. Many rhizoids had branching hyphae at their distal end. Rarely, several rhizoids had darkly stained cylindrical and oval, softly pigmented vesicles (Figs. 2c and 2d). Mycorrhizal colonization was calculated between 59-84%. The presence of mycelium in the thallus tissue was examined in six populations. Fungus occupied the storage zone's central mid-rib region in all the studied samples. The remainder of the storage zone, the photosynthetic tissue and the wings were uninvaded (Fig. 2e). Only a few center cells in the midrib area of the plant were tightly packed with fungal threads (Fig. 2f), whereas cells surrounding the central cells had hyphae dispersed throughout them. In certain cells, arbuscules were also observed (Fig. 2g). Notably, these associations showed higher colonization of fungal hyphae in smooth-walled rhizoids compared to tuberculated rhizoids, enabling their spread into the gametophyte parenchyma via plasmodesmata, by passing gametophytic intercellular spaces typical of liverworts, except Haplomitriopsida. In terms of structural patterns, liverworts displayed similarities with both Arum and Paris types found in arbuscular mycorrhizal (AM) fungi of flowering plants (Rimington et al. 2018). Glomus aureum synonym Dominikia aurea (Oehl. & Sieverding.) Blasz., Chwat, was isolated using trap culture.

Enzyme activities

Ascorbate peroxidase (APOX)

APOX were estimated between 1.92-2.09 UA/mg protein and it displayed a strong positive correlation (0.90) with mycorrhizal colonization (Graph 2). APOX is involved in the ascorbate-glutathione cycle, which is crucial for scavenging reactive oxygen species, indicating an efficient antioxidant system during mycorrhizal colonization. This enzyme plays a role in the ascorbate-glutathione cycle and is important for ROS detoxification. Yadav et al. (2022) showed similar APOX activity (2.5 UA per mg protein) in Dumortiera hirsuta from Khasi Hills, Shillong, Meghalaya. This finding
suggests that liverworts possess the capacity to scavenge reactive oxygen species (ROS) using APOX.

Catalase (CAT)

CAT was calculated 0.01 UA/mg protein, as this amount was very little, so correlation was not calculated in catalase. Sharma et al. (2015) conducted research comparing catalase activity in two liverwort species, *Plagiochasma* sp. and *Pellia* sp. They found that *Plagiochasma* sp. had significantly higher catalase activity (0.014UA/mg Prot. g⁻¹ FW) compared to *Pellia* sp. (0.0045 UA/mg Prot. g⁻¹ FW). Our results also confirmed the similar catalase activity in liverworts.

Glutathione reductase (GR)

GR was in the 0.05-0.01 UA/mg protein range and showed a moderate positive correlation (0.68) with mycorrhizal colonization (Graph 3). GR plays a role in the regeneration of reduced glutathione, suggesting the importance of glutathione-related antioxidant processes in mycorrhizal liverworts. Arbuscular mycorrhizal (AM) symbiosis is a mutualistic association between plants and fungi, specifically the arbuscular mycorrhizal fungi (AMF).

Guaiacol peroxidase (GPOX)

GPOX was in the range of 0.24-0.26 UA/mg protein

Figure 1. 2a-i. AM associations in *Asterella multiflora*. Fig. a: Thallus of *Asterella multiflora*. Fig. b-d: Smooth walled rhizoids showing fungal hyphae and vesicles of different shapes, cylindrical (Fig. c) and spherical (Fig. d) and vesicles. Fig. e: V. S of thallus showing fungal central, compactly filled cells and cells having intermingled hyphae. Fig. g: A magnified view of cells of midrib region showing arbuscules. h: Trap culture of AM fungi in *Sorghum vulgare*. Fig. i: *Glomus aureum* (Oehl & Sieverd) isolated from the trap culture. (Bar: 20µm).
and exhibited a moderate positive correlation (0.83) with mycorrhizal colonization (Graph4). GPOX is involved in the reduction of hydrogen peroxide, indicating a potential role in antioxidant defense during mycorrhizal colonization. It's essential to acknowledge the reference values provided by Sharma et al. in 2015, which reported higher guaiacol peroxidase activity (0.93 UA/mg protein) in Plagiochasma appendiculatum and 0.12 UA/mg protein in another liverwort, Pellia endiviifolia. These differences between our results and those of the previous study could be attributed to various factors, including variations in experimental conditions, environmental factors, or genetic diversity among liverwort populations. It's important to note that many variables can influence enzyme activity, and the specific conditions under which the enzyme is assayed can significantly impact the activity.

Table 2. Table showing Protein, ascorbate peroxidase activity (APOX), catalase (CAT), superoxide dismutase (SOD), glutathione reductase activity (GR) and guaiacol peroxidase (GPOX) in different populations of Asterella multiflora.

<table>
<thead>
<tr>
<th>Population</th>
<th>Protein mg/gm F.W.</th>
<th>APOX UA/mg protein</th>
<th>CAT UA/mg protein</th>
<th>SOD UA/mg protein</th>
<th>GR UA/mg protein</th>
<th>GPOX UA/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.28</td>
<td>1.99</td>
<td>0.013</td>
<td>0.152</td>
<td>1.18</td>
<td>0.26</td>
</tr>
<tr>
<td>2</td>
<td>5.38</td>
<td>2.09</td>
<td>0.014</td>
<td>0.055</td>
<td>1.19</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>5.47</td>
<td>2.15</td>
<td>0.014</td>
<td>0.104</td>
<td>1.01</td>
<td>0.26</td>
</tr>
<tr>
<td>4</td>
<td>5.18</td>
<td>1.92</td>
<td>0.014</td>
<td>0.094</td>
<td>1.18</td>
<td>0.26</td>
</tr>
<tr>
<td>5</td>
<td>5.4</td>
<td>2.11</td>
<td>0.014</td>
<td>0.155</td>
<td>1.14</td>
<td>0.24</td>
</tr>
<tr>
<td>6</td>
<td>5.45</td>
<td>2.16</td>
<td>0.014</td>
<td>0.104</td>
<td>1.01</td>
<td>0.26</td>
</tr>
<tr>
<td>Mean</td>
<td>5.36</td>
<td>2.07</td>
<td>0.013833</td>
<td>0.110667</td>
<td>1.118333</td>
<td>0.255</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.110454</td>
<td>0.095289</td>
<td>0.000408</td>
<td>0.037787</td>
<td>0.085654</td>
<td>0.008367</td>
</tr>
</tbody>
</table>

Graph 1. Graph showing Protein, ascorbate peroxidase activity (APOX), glutathione reductase activity (GR), superoxide dismutase (SOD) and guaiacol peroxidase (GPOX) in Asterella multiflora.
Superoxide dismutase (SOD)

SOD activities were calculated between 0.05-0.015 UA/mg protein in six populations of Asterella aungusta and it showed a moderate positive correlation (0.88) with mycorrhizal colonization (Graph 5). SOD is an enzyme that helps detoxify superoxide radicals, indicating a potential role of mycorrhizal colonization in oxidative stress regulation. Mycorrhizal plants often show increased activities of antioxidant enzymes, including SOD. The extract of liverwort Plagiochasma sp. used by the Gaddi tribe in the Kangra Valley for treating skin diseases is of particular interest. The study found that this extract not only prevents lipid peroxidation but also

Graph 3. Scattered diagram showing relation between protein and percent mycorrhizal colonization (PMC) in Asterella multiflora.

Graph 2. Scattered diagram showing the relation between Ascorbate Peroxidase and percent mycorrhizal colonization (PMC) in Asterella multiflora.
increases SOD and CAT (Catalase) activity (Kumar et al., 2008). The increase in SOD activity suggests that the extract enhances the detoxification of superoxide radicals, contributing to its antioxidant properties. This is highly relevant in the context of skin health, as oxidative stress is a common factor in skin diseases.

While the direct impact of AM symbiosis on antioxidant capacity is not fully understood, there are several indirect ways in which it may influence plant antioxidant defenses:

**Nutrient Uptake and Plant Health**

AMF enhances the absorption of nutrients,

Graph 4. Scattered diagram showing the relation between Glutathione Reductase and percent mycorrhizal colonization (PMC) in *Asterella multiflora*.

Graph 5. Scattered diagram showing the relation between guaiacol peroxidase and percent mycorrhizal colonization (PMC) in *Asterella multiflora*.
particularly phosphorus, from the soil and deliver them to the plant. Improved nutrient availability can contribute to overall plant health and vitality. Healthy plants are better equipped to produce and maintain their antioxidant defense systems (Marschner & Dell, 1994).

**Stress Tolerance**

AM symbiosis has been associated with increased tolerance to various environmental stresses, such as drought, salinity, and heavy metal toxicity. Stress conditions often lead to the production of reactive oxygen species (ROS) in plants. Antioxidant enzymes like superoxide dismutase, catalase, and peroxidase help mitigate the harmful effects of ROS. AMF-mediated stress tolerance can indirectly enhance the antioxidant capacity of plants (Singh et al., 2011; Mitra et al., 2021).

**Enhanced Secondary Metabolites**

AM symbiosis can stimulate the production of secondary metabolites in plants, including phenolics and flavonoids. Some of these compounds have antioxidant properties and contribute to the plant's ability to neutralize ROS (Balestrasse and Tomaro, 2001; Yeshi et al., 2022).

**Induction of Defense Pathways**

The interaction between AMF and plant roots can activate various plant defense pathways. This includes the induction of systemic acquired resistance (SAR) and the production of phytohormones such as salicylic acid and jasmonic acid. These signaling pathways play a role in regulating antioxidant enzyme activities (Kadam et al., 2020).

**Reduced Oxidative Stress**

By improving nutrient acquisition and overall plant health, AM symbiosis may help minimize nutrient deficiencies that can lead to oxidative stress. This, in turn, can contribute to a balanced antioxidant defense system in plants (Mitra et al., 2021).

Mycorrhizal symbiosis can enhance the activities of antioxidant enzymes, including in plants, which helps detoxify ROS. When plants receive nutrients through mycorrhizal networks, their overall antioxidant enzyme activity can increase, so, the enhanced enzyme activity can be attributed to improved overall health. Moreover, mycorrhizal symbiosis can enhance the antioxidant defense system of plants, as it often enhances plant tolerance to various stresses, such as drought, salinity, and diseases. In response to stress, plants may upregulate the production of enzymes such as SOD, GPOX, CAT, APOX and GR, to cope with the increased oxidative stress. In summary, *Glomus aureum* colonization in liverworts appears to be associated with a robust antioxidant defense system, including enzymes such as SOD, GPOX, CAT, APOX, and GSH, indicating a symbiosis-induced increase in liverwort stress tolerance. These antioxidants likely play a crucial role in stress tolerance and overall plant health during mycorrhizal interactions.
Conclusion
This study sheds light on the understudied AM symbiosis in liverworts and its impact on antioxidant potential. The positive correlation between Glomus aureum colonization and enhanced antioxidants emphasizes the ecological importance of this symbiotic relationship. The findings contribute valuable insights into the intricate interconnections between plants, fungi, and their environment, underscoring the need for further research in this area. Understanding the dynamics of AM symbiosis in liverworts enriches our knowledge of plant-fungal interactions and provides a basis for exploring innovative agricultural and environmental applications.

Acknowledgements
The authors express sincere gratitude to the Department of Botany at the University of Jammu for the invaluable support provided during the time of conducting research in the laboratory.

Conflict of Interest
The authors declare no conflict of interest.

References

https://doi.org/10.1007/978-94-017-1284-28

https://doi.org/10.1007/s40011-014-0373-0

https://doi.org/10.4161/psb.6.2.14146

https://doi.org/10.1016/j.jep.2006.02.007


https://www.cabidigitiallibrary.org/doi/full/10.5555 /20113310904

